Effect of Chitosan on Cucumber Plants: Suppression of *Pythium aphanidermatum* and Induction of Defense Reactions

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Supported by the conseil des recherches en pêches et agro-alimentaire (CORPAQ).
Accepted for publication 19 October 1993.

ABSTRACT

El Ghaouth, A., Arul, J., Grenier, J., Benhamou, N., Asselin, A., and Bélanger, R. 1994. Effect of chitosan on cucumber plants: Suppression of *Pythium aphanidermatum* and induction of defense reactions. Phytopathology 84:313-320.

Biological activity of chitosan on Pythium rot of cucumber was investigated. Growing cucumber plants in the presence of chitosan (100 or 400 μg/ml) controlled root rot caused by *Pythium aphanidermatum* and triggered several host defense responses, including the induction of structural barriers in root tissues and the stimulation of antifungal hydrolases (chitinase, chitosanase, and β-1,3-glucanase) in both the roots and leaves. Whereas chitosan did not cause any apparent phytotoxicity to cucumber plants, it adversely affected the growth of *P. aphanidermatum*. A close examination of hyphal cells revealed that chitosan caused wall loosening, vacuolation, and, in some cases, protoplasm disintegration. This may, in part, explain the limited ability of the pathogen to colonize root tissues in the presence of chitosan. Ultrastructural study of root tissue from chitosan-treated plants showed that fungal cells were mainly restricted to root surfaces. The interplay of the antifungal and eliciting properties of chitosan makes chitosan a potential antifungal agent for the control of root rot of cucumber caused by *P. aphanidermatum*.

Additional keywords: *Cucumis sativus*, elicitor, glucanohydrolases, β-(1,4)-glucosamine polymer.

Pythium species are soilborne pathogens that limit yield on a wide range of greenhouse-grown crops (17,21,28,29), including English cucumber (*Cucumis sativus* L.). Although *Pythium* species are considered damping-off pathogens, they can cause crown, root, and lower stem rot of mature plants, which often leads to wilting and ultimately to plant death especially during warm sunny days (23). Of the *Pythium* species isolated from mixes used for cucumber seedling propagation, *Pythium aphanidermatum* (Edson) Fitzp. has been reported to be the most aggressive (17). In Québec, *P. aphanidermatum* causes major losses in greenhouse cucumber production, and the problem is further accentuated by conditions in hydroponically grown cultures that favor the proliferation of the fungus (30). Diseases caused by *Pythium* species are of particular importance because there are no commercially available *Pythium*-resistant cucumber cultivars nor are there any fungicides registered for use in hydroponic systems (34).

Recently, two approaches, UV irradiation (29) and potassium silicate amendment of nutrient solutions (10), have been proposed to control *Pythium* infections in hydroponic culture systems. Although these approaches have shown promising results, they are still at the experimental stage and are not widely used commercially. Another potential approach involves the use of the natural bioactive substance chitosan, which inhibits fungal growth and also activates defense mechanisms of plants (2,14,24). This nontoxic β-(1,4)-glucosamine polymer is obtained from the chitin of fungal walls and arthropod exoskeletons that has been chemically deacetylated to provide more than 70% free amino groups (18). The polycationic nature of chitosan provides the basis for its physico-chemical and biological function. Chitosan inhibits a number of pathogenic fungi (2,13), including several soilborne pathogens such as *Fusarium oxysporum, Rhizoctonia solani,* and *P. paroecandrum* (4,24,25,31). Leuba and Stossel (25) reported that chitosan at pH 5.8 (when most amino groups are protonated) induced massive leakage of UV-absorbing materials in *P. paroecandrum*.

Chitosan also can induce a multitude of biological processes in plant tissues, including the stimulation of chitinases (14,26), accumulation of phytoalexins (24,33), synthesis of proteinase inhibitors (33), and increased lignification (27). Growing plants in the presence of chitosan could affect pathogenic fungi by direct antimicrobial interaction and/or by triggering the plants' natural defense mechanisms and consequently helping the tissue restrict fungal colonization. This dual function could be exploited for the control of greenhouse diseases.

The objectives of this research were to first determine the effect of chitosan amendment on fungal growth and development of Pythium root rot of cucumber plants in hydroponic systems, second delineate some of the biological events associated with chitosan cucumber interaction, and third assess the direct inhibitory effect of chitosan on *P. aphanidermatum*.

MATERIALS AND METHODS

Reagents and fungi. Crab-shell chitosan was obtained from ICN Biochemical Inc. (Cleveland), milled to a fine powder, and purified as described previously (13,14). A chitosan solution was prepared by dissolving chitosan in 0.04 N HCl and adjusting the pH to 5.6 with 2 N KOH. *P. aphanidermatum* was obtained from J. Menzies (Agriculture Canada, Agassiz, BC), and maintained on potato-dextrose agar (PDA). Inoculum suspensions, containing mycelium plus oospores, were made by homogenizing, four 6-day-old *P. aphanidermatum* cultures in 20 ml of sterile distilled water with a Waring blender at low speed for 2 min. The inoculum density was adjusted with sterile distilled water to 10^8 cfu/ml. Preliminary studies have shown that this inoculum density causes high disease incidence. The chemicals used for electrophoresis and protein molecular mass markers were purchased from Bio-Rad (Mississauga, Ontario, Canada). Calcofluor White M2R (C.I. 4062) and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis). Glutaraldehyde and osmium tetroxide were obtained from JBEM Chemical Co. (Pointe-Claire, Québec), and tetrachlorauric acid was obtained from BDH

Chemicals (Montreal). All other chemicals for electron microscopy were purchased from Sigma.

Plant material. Seeds of cucumber cultivar Corona (De Ruiter Seeds Inc., Columbus, OH) were sown in LC-1 Horticubes (Smithers-Oasis, Kent, OH) and fertilized weekly with a nutrient solution of 7-11-27 ($N\text{-}P_2O_5\text{-}K_2O$) for 3 wk under greenhouse conditions. Plants with the second leaves fully expanded were individually transferred to 5-L plastic tanks containing 4.5 L of autoclaved nutrient solution (11). In each tank, six plants were spaced at 10-cm intervals and secured with perforated plastic lids. The nutrient solution in the tanks was adjusted to pH 5.6 with 2 N KOH, aerated with aquarium pumps, and changed every 5 days. The electrical conductivity of the solution ranged from 1.7 to 2.0 mS/cm depending on the concentration of chitosan added. Plants were kept at 22–25 C and were supplemented with 16 h of cool-white fluorescent light (145 $\mu E\ m^{-2}\ s^{-1}$).

Effect of chitosan. *Experiment 1.* Cucumber plants were grown in the nutrient solution supplemented with chitosan. Chitosan was dissolved in 0.04 N HCl, and the pH was adjusted to 5.6 with 2.0 N KOH. The chitosan solution was added to the nutrient solution to obtain chitosan concentrations of 0, 100, 200, or 400 μg/ml. The solutions were autoclaved and subsequently dispensed in 5-L tanks. Treatments consisted of three tanks each that contained six plants. Treatments were arranged in a randomized complete block design and replicated four times. The experiment was maintained for 2 wk after the transfer of the plants to the tanks. The experiment was performed twice. Plants were evaluated daily for apparent symptoms of phytotoxicity. Samples of the nutrient solutions were collected every 2 days and assayed for protein and chitosan breakdown. The amount of protein leaked into the nutrient solution was determined by the method of Bradford (7). The electrophoretic profiles of chitosan in the nutrient solutions over a period of 14 days were determined by the method of Audy and Asselin (3).

Leaf and root samples also were collected every 2 days over a period of 14 days. At each sampling date, four replicate plants per treatment were used. Intercellular fluid extracts from leaf samples were obtained by vacuum infiltration followed by low-speed centrifugation (20). Freshly collected leaves were cut into pieces 5–7 cm^2, washed with sterile water, and infiltrated in vacuo with 50 mM sodium phosphate (pH 5.0), with gentle agitation for three periods of 50 s each. Leaf sections were gently blotted dry with paper towels, rolled up, and placed in an 11-ml polypropylene Econocolumn (Bio-Rad) inserted into a 1.5-ml Eppendorf microcentrifuge tube. The Econocolumn containing leaf sections was placed in a 30-ml Corex centrifugation tube (Fisher Scientific, Montreal). After centrifugation at 3,000 *g* for 10 min at 4 C, the fluid in the bottom of the Eppendorf tube was recovered and used immediately or frozen at −20 C. A portion of each root sample was processed for electron microscopy whereas the remaining part was used for crude enzyme preparation. Root tissues were homogenized with a prechilled mortar and pestle in 50 mM sodium phosphate buffer (pH 5.0) (1:1, w/v). The homogenate was centrifuged at 4 C (15 min, 10,000 *g*), and the supernatant was used as the crude enzyme preparation. Protein content in supernatants and leaf intercellular fluid was determined with the Bio-Rad protein assay kit.

Experiment 2. Cucumber plants were transferred to plastic tanks containing the nutrient solution amended with purified chitosan at 0 or 400 μg/ml. After 24 h, plants were inoculated by adding 3 ml of an inoculum suspension at 10^8 cfu/ml to the nutrient solutions. As the control, sterile potato-dextrose broth was added. Four replicates of three tanks containing six plants each were used for each treatment. Treatments were arranged in a randomized complete block design, and the experiment was performed twice. The experiment was maintained for 2 wk. The presence of *P. aphanidermatum* propagules in the nutrient solutions was determined 7 days after inoculation. Serial dilutions of samples from each treatment were plated onto a selective medium (22). Cucumber plants were monitored periodically for wilt symptoms and plant death. Root samples were collected from four replicate plants per treatment every 2 days during a period of 14 days after inoculation. A portion of the root system was processed for electron microscopy, and the remaining part was used for crude enzyme preparation as described above.

Tissue processing for transmission electron microscopy (TEM). Root samples were severed and held in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h at 4 C, postfixed for 1 h at 4 C in 1% osmium tetroxide in the same buffer, dehydrated in a graded ethanol series, and then embedded in Epon 812 (BDH Chemicals, Montreal). An average of four blocks per treatment per time interval was sectioned. Ultrathin sections (0.1 μm), collected on formvar-coated nickel grids (200 mesh) were contrasted with uranyl acetate and lead citrate before examination with a JEOL 1200 EX (Tokyo) transmission electron microscope at 80 kV.

Detection of chitinase, chitosanase, and β-1,3-glucanase activities after polyacrylamide gel electrophoresis (PAGE). Sample preparation and protein separation after PAGE under native conditions at pH 4.3 (Reisfeld system) or at pH 8.9 (Davis system) or under denaturing sodium dodecyl sulfate (SDS)-PAGE were performed as previously described (14,20). Chitinase and chitosanase activities after SDS-PAGE were detected by Calcofluor White M2R staining after hydrolysis of 0.01% (w/v) glycol chitin or glycol chitosan as substrate in the gel matrix (14,20,32), respectively. Chitosanase activities also were detected by staining the intact glycol chitosan with Coomassie blue (20). After SDS-PAGE, proteins were renatured by incubating the gel overnight at 37 C in 200 ml of 100 mM sodium acetate buffer (pH 5.0) with 1.0% (v/v) purified Triton X-100. β-1,3-glucanase activity was analyzed after native PAGE, pH 8.9 or 4.3, after lysis of baker's yeast glucan (0.6 μg/ml) as substrate. The alkali-soluble glucan from the cell wall of *Saccharomyces cerevisiae* (strain W303a) was extracted according to the method of Cabib and Bowers (9). For gels run at pH 8.9, the soluble glucan was incorporated in the gel matrix, whereas in gels run at pH 4.3, β-1,3-glucanase activity was analyzed by transferring proteins separated in polyacrylamide gel to a 7.5% (w/v) overlay of polyacrylamide gels containing alkali-soluble yeast glucan at 0.6 μg/ml. The transfer of proteins to the overlay gel was done by blotting (20). Staining of native gels was done with aniline blue as described for tobacco pathogenesis-related proteins (12).

Antifungal activity of chitosan. Antifungal properties of chitosan against *P. aphanidermatum* were determined in shake-flask cultures of plant nutrient solution containing 10% (w/v) sucrose and amended with different concentrations of chitosan. Chitosan, processed as previously described, was added to the plant nutrient solution (pH 5.6) containing 10% (w/v) sucrose to obtain chitosan concentrations of 0, 100, 200, or 400 μg/ml. The solution was dispensed into 250-ml Erlenmeyer flasks and autoclaved. Each flask was seeded with two 6-mm-diameter mycelial plugs taken from the edges of actively growing *P. aphanidermatum* colonies (4 days old on PDA). Four replicates of six flasks were used for each concentration of chitosan, and the flasks were incubated for 5 days at 24 C with orbital shaking (150 rpm). At the end of the incubation period, the mycelial dry weight was determined. The treatments were arranged in a randomized design, and the test was conducted twice.

To further characterize the antifungal activity of chitosan, a study was conducted on PDA plates containing chitosan at 0 and 400 μg/ml prepared as previously described (14). PDA plates were seeded with 6-mm-diameter mycelial plugs taken from the edges of 4-day-old *P. aphanidermatum* cultures. After 20 h of incubation at 24 C, mycelial samples were collected and processed for TEM. For each treatment, five samples were collected from five replicate plates.

TEM and cytochemical labeling of cellulose. Mycelial samples were fixed, postfixed, dehydrated, and embedded in Epon 812 following the procedure described earlier. Ultrathin sections (0.1 μm), collected on formvar-coated nickel grids, were contrasted with uranyl acetate and lead citrate before examination with a JEOL 1200 EX transmission electron microscope at 80 kV or were processed for cytochemical labeling prior to examination. The colloidal gold suspension was prepared following the method

of Frens (19). The distribution of cellulose within the cell wall of *P. aphanidermatum* was studied with an exoglucanase-gold complex, prepared as described by Benhamou et al (5).

First, ultrathin sections were floated for 5 min on a drop of phosphate-buffered saline polyethylene glycol (PBS-PEG; pH 6.0) and, thereafter, were incubated on a drop of the enzyme-gold complex for 30 min in a moist chamber. After washing thoroughly with PBS (pH 7.4) and rinsing with double distilled water, grids were stained with uranyl acetate and lead citrate. An average of four blocks per treatment was sectioned and at least five serial sections from each block were examined under an electron microscope. The experiment was conducted four times. Control tests included: 1) incubation with the exoglucanase-gold complex to which was previously added β-(1,4)-glucan from barley at 1.0 mg/ml; 2) incubation with a bovine serum albumin–gold complex; and 3) incubation with stabilized gold suspension.

RESULTS

Effect of chitosan on cucumber. Cucumber plants grown in the presence of different concentrations of chitosan did not show any apparent symptoms of phytotoxicity during the course of the experiment even at the highest chitosan concentration. Treatment with chitosan, however, appeared to slightly affect the morphology of secondary roots. Plants in chitosan amended nutrient solution had secondary roots that were shorter and thicker than those of nontreated plants. These changes did not visibly influence foliar development, as these plants appeared more vigorous than the nontreated control plants. Analysis of samples taken at different time intervals from the plant growth medium indicated that chitosan did not induce any significant increase in leakage of proteins. The amount of proteins released from nontreated controls with respect to time was almost similar to that observed from plants treated with chitosan at 400 μg/ml. After 2, 6, and 12 days of growth, the amount of proteins released from plants treated with chitosan at 400 μg/ml (6.2, 8.7, and 10.3 μg of protein per milliliter) was slightly higher than that of controls (5.6, 8.2, and 9.6 μg of protein per milliliter). Increasing the concentration of chitosan from 100 to 400 μg/ml did not result in any marked increase in leakage of proteins.

To determine whether chitosan was hydrolyzed during plant growth, samples from the nutrient solution were subjected to SDS-PAGE and analyzed for chitosan oligosaccharides. The electrophoretic profiles of samples from nutrient solution containing chitosan revealed only one major diffuse band located at the top of the gel (*data not shown*). This band was similar to the one detected in the standard chitosan sample.

Effect of chitosan on chitinase, chitosanase, and β-1,3-glucanase activities. Root and leaf intercellular fluid extracts of cucumber plants grown in concentrations of chitosan at 0, 100, 200, or 400 μg/ml were first analyzed for chitinase and chitosanase activity after SDS-PAGE. Chitinase and chitosanase activities were visualized as dark (nonfluorescent) bands against a UV-fluorescent background. Extracts of root tissue from cucumber plants grown in concentrations of chitosan at 0, 100, or 400 μg/ml for 1, 7, and 14 days exhibited one major chitinolytic band at approximately 35 kDa with increased activity at higher chitosan concentrations (Fig. 1A, lanes 2 and 3, 5 and 6, 8 and 9). This same band was also present in the control, but its activity was low (Fig. 1A, lanes 1, 4, and 7). The intensity of the chitinolytic band detected in root extracts from plants grown in the presence of chitosan remained at an elevated level for 14 days (Fig. 1A, lanes 2 and 3 versus 8 and 9). Growing cucumber plants in nutrient solutions amended with chitosan also stimulated chitinase activity in the leaves (Fig. 1B). In leaf intercellular fluid extracts from treated plants, one large band with chitinolytic activity at 24 kDa was detected (Fig. 1B, lanes 2 and 3, 5 and 6, 8 and 9). The same activity was present in smaller amounts in leaf intercellular fluid from control plants (Fig. 1B, lanes and 4), except in the sample taken after 14 days of growth, in which the activity of the chitinase band was similar to that from chitosan-treated plants (Fig. 1B, lane 7 versus 8 and 9).

An increase in chitosanase activity also was detected in both root and leaf intercellular fluid extracts of plants treated with chitosan. Root extracts of plants grown in concentrations of chitosan at 0, 100 or 400 μg/ml for 1, 7, or 14 days showed two major bands, with chitosanase activity at approximately 14 and 10 kDa and two faint ones at 22 and 27 kDa (Fig. 2A, lanes 2 and 3, 5 and 6, 8 and 9), whereas extracts from control plants exhibited only one band at 14 kDa with low chitosanase activity (Fig. 2A, lanes 1, 4, and 7). The activity of the bands at 14 and 10 kDa was stimulated by chitosan with a greater effect at higher concentrations, whereas that of the minor bands at 22 and 27 kDa appeared less affected by the increase in chitosan concentration (Fig. 2A, lanes 2, 5, and 8 versus lanes 3, 6, and 9). The level of activity exhibited by the bands at 14 and 10 kDa remained high during the course of the experiment. A similar increase in chitosanase activity also was observed with leaf intercellular fluid extracts (Fig. 2B). Four bands with chitosanase activity at 18, 14, 10, and 8 kDa were detected in leaf intercellular fluid extracts of plants grown in the presence of concentrations of chitosan at 100 or 400 μg/ml for 1, 7, and 14 days (Fig. 2B, lanes 2 and 3 , 5 and 6, 8 and 9). Only the activity at 18 kDa was present in leaf intercellular fluid extracts of control plants (Fig. 2B, lanes 1, 4, and 7).

Leaf intercellular fluid and root extracts of cucumber plants also were analyzed for β-1,3-glucanase activity under native conditions for acidic (Davis system) and basic (Reisfeld system) proteins. In the Davis system, root extracts of plants grown in the presence of chitosan at 100 or 400 μg/ml for 1 or 7 days exhibited one major band (top of the gel) and two minor bands with β-1,3-glucanase activity (Fig. 3, lanes 8 and 9, 11 and 12), whereas only the upper band was present in the controls (Fig. 3, lanes 7 and 10). Among the acidic glucanase isoforms present in the roots, only the lower band appeared to increase in intensity with a higher chitosan concentration (Fig. 3, lane 8 versus 9 and 11 versus 12). A similar glucanase isozyme profile was observed

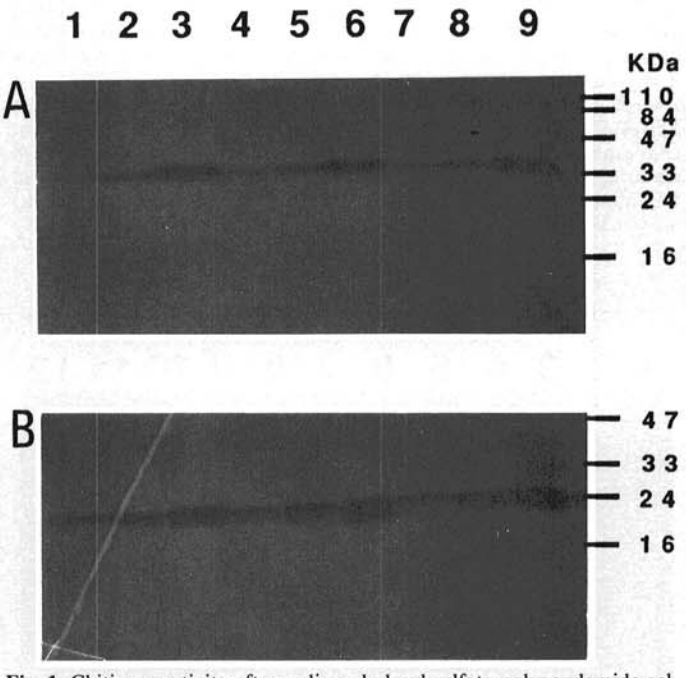

Fig. 1. Chitinase activity after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Extracts (20 μl) of root (28 μg of protein) and leaf intercellular fluid (3.0 μg of protein) from cucumber plants grown in the presence of chitosan at 0 (lanes 1, 4, and 7); 100 (lanes 2, 5, and 8); or 400 μg/ml (lanes 3, 6, and 9) for 1, 7, and 14 days, respectively, were assayed for chitinase. Extracts were subjected to SDS-PAGE in a polyacrylamide slab gel containing 0.01% (w/v) glycol chitin as substrate. Chitinase activity was visualized by staining with Calcofluor White M2R. **A and B** show chitinase activities in root and leaf intercellular fluid, respectively. Molecular mass markers (kiloDaltons) are indicated on the right.

with leaf intercellular fluid extracts of chitosan-treated plants (Fig. 3, lanes 2 and 3, 5 and 6). No β-1,3-glucanase activity could be detected in leaf intercellular fluid extracts from control plants (Fig. 3, lanes 1 and 4). In the Reisfeld system, designed to separate basic proteins, only one band with β-1,3-glucanase activity was present in root and leaf intercellular fluid extract of chitosan-treated plants (*data not shown*). This band appears to correspond to the upper acidic band detected in the Davis system.

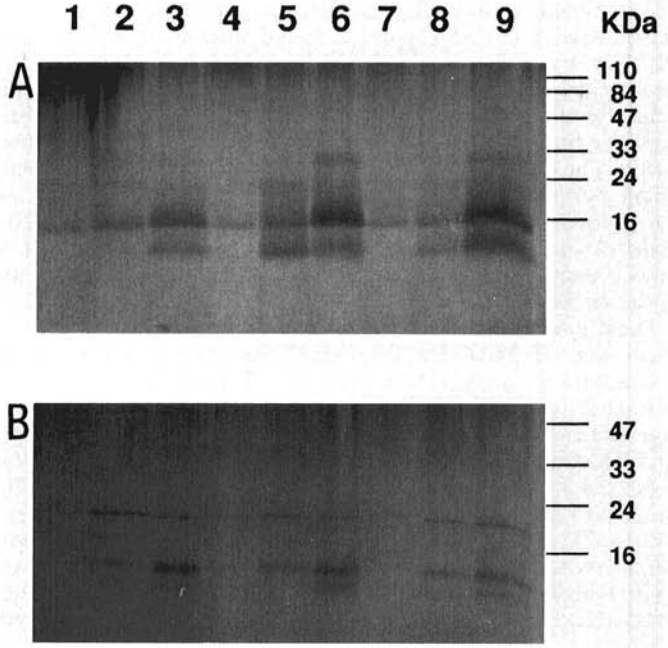

Fig. 2. Chitosanase activity after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Extracts (20 μl) of root (28 μg of protein) and leaf intercellular fluids (3.0 μg of protein) from cucumber plants grown in the presence of chitosan at 0 (lanes 1, 4, and 7); 100 (lanes 2, 5, and 8); or 400 μg/ml (lanes 3, 6, and 9) for 1, 7, and 14 days, respectively, were assayed for chitosanase. Extracts were subjected to SDS-PAGE in a polyacrylamide slab gel containing 0.01% (w/v) glycol chitosan as substrate. Chitosanase activity was visualized by staining with Calcofluor White M2R. **A and B** show chitosanase activities in root and leaf intercellular fluid, respectively. Molecular mass markers (kiloDaltons) are indicated on the right.

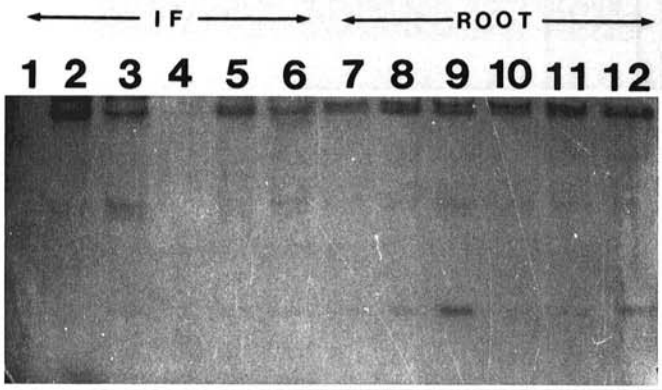

Fig. 3. β-1,3-glucanase activity after native polyacrylamide gel electrophoresis (PAGE) specific for acidic proteins. Root extracts from cucumber plants grown for 1 and 7 days in the presence of chitosan at 0 (lanes 7 and 10); 100 (lanes 8 and 11); or 400 μg/ml (lanes 9 and 12), respectively, were subjected to native PAGE in a 15% (w/v) polyacrylamide gel containing yeast glucan (0.6 μg/ml) as substrate. Leaf intercellular fluid (IF) extracts from the same plants treated with chitosan at 0 (lanes 1 and 4); 100 (lanes 2 and 5); or 400 μg/ml (lanes 3 and 6) for 1 and 7 days also were analyzed for β-1,3-glucanase activity. Glucanase activity was revealed by aniline blue.

Effect of chitosan on symptoms. Chitosan treatment was effective in controlling the incidence of root rot (Fig. 4). Cucumber plants grown in the presence of chitosan (400 μg/ml) and inoculum of *P. aphanidermatum* remained healthy and did not exhibit symptoms of wilting throughout the experiment (14 days). Their root systems were well developed and did not show any symptoms of decay. Plants grown in the presence of inoculum alone (control plants) started showing symptoms of wilting within 24 h after inoculation. By day three, control plants were either dead or had reached an advanced stage of wilting and root decay. Analysis of nutrient solutions revealed that *P. aphanidermatum* was present in chitosan-treated and nontreated nutrient solutions. However, *P. aphanidermatum* recovered from chitosan-treated solution displayed attenuated and abnormal growth characterized by excessive branching.

Root samples from inoculated plants treated with chitosan (0 or 400 μg/ml) were analyzed for glucanohydrolase activity. In root extracts, an increase in chitinase, chitosanase, and β-1,3-glucanase activities was observed after inoculation with *P. aphanidermatum*. The level of increase was more pronounced in inoculated chitosan-treated plants than in inoculated control plants (*data not shown*). The activity detected in inoculated chitosan-treated plants was comparable to that observed in chitosan-treated plants (*data not shown*).

Cytological study of the effect of chitosan on symptoms. Examination of ultrathin sections taken 4 days after inoculation from infected roots of control plants revealed a massive colonization of the cortex region, endodermis, and vascular elements (Fig. 5A). Pathogen ingress was followed by marked alteration of the host wall, such as swelling and disintegration (Fig. 5A–B, arrows). Fungal growth occurred intracellularly and intercellularly, causing extensive cell damage and even death. Various host reactions, which have been previously described by Chérif et al (11) were observed in the vicinity of the vascular region. These reactions were characterized mainly by the occlusion of some parenchyma cells with an electron-opaque, amorphous material (Fig. 5A). They did not, however, halt fungal ingress toward the vascular stele.

In root tissues from plants grown in the presence of chitosan, fungal cells were restricted mainly to the root surface. There was little evidence of successful fungal penetration of the outer epi-

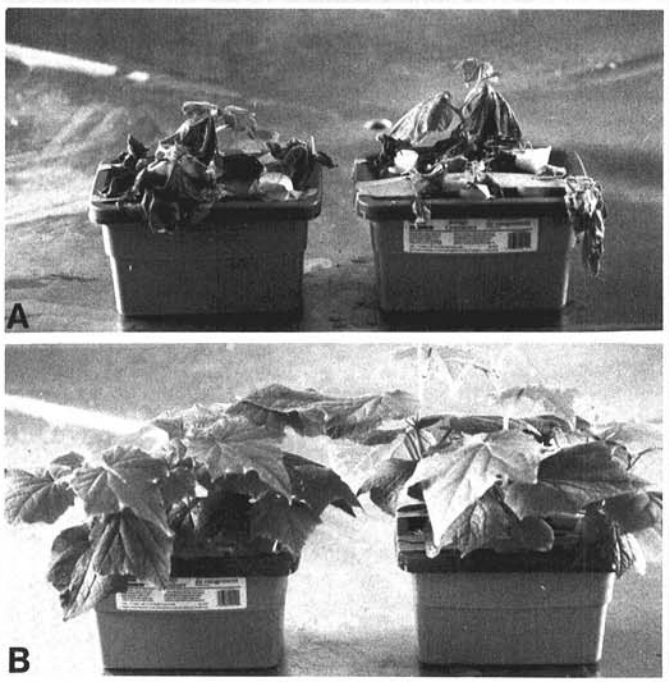

Fig. 4. Effect of chitosan on *Pythium aphanidermatum* infection of cucumber plants. **A,** Plants grown for 3 days in nutrient solution inoculated with *P. aphanidermatum*. **B,** Plants grown for 7 days in nutrient solution supplemented with 400 μg/ml of chitosan and inoculated with *P. aphanidermatum*.

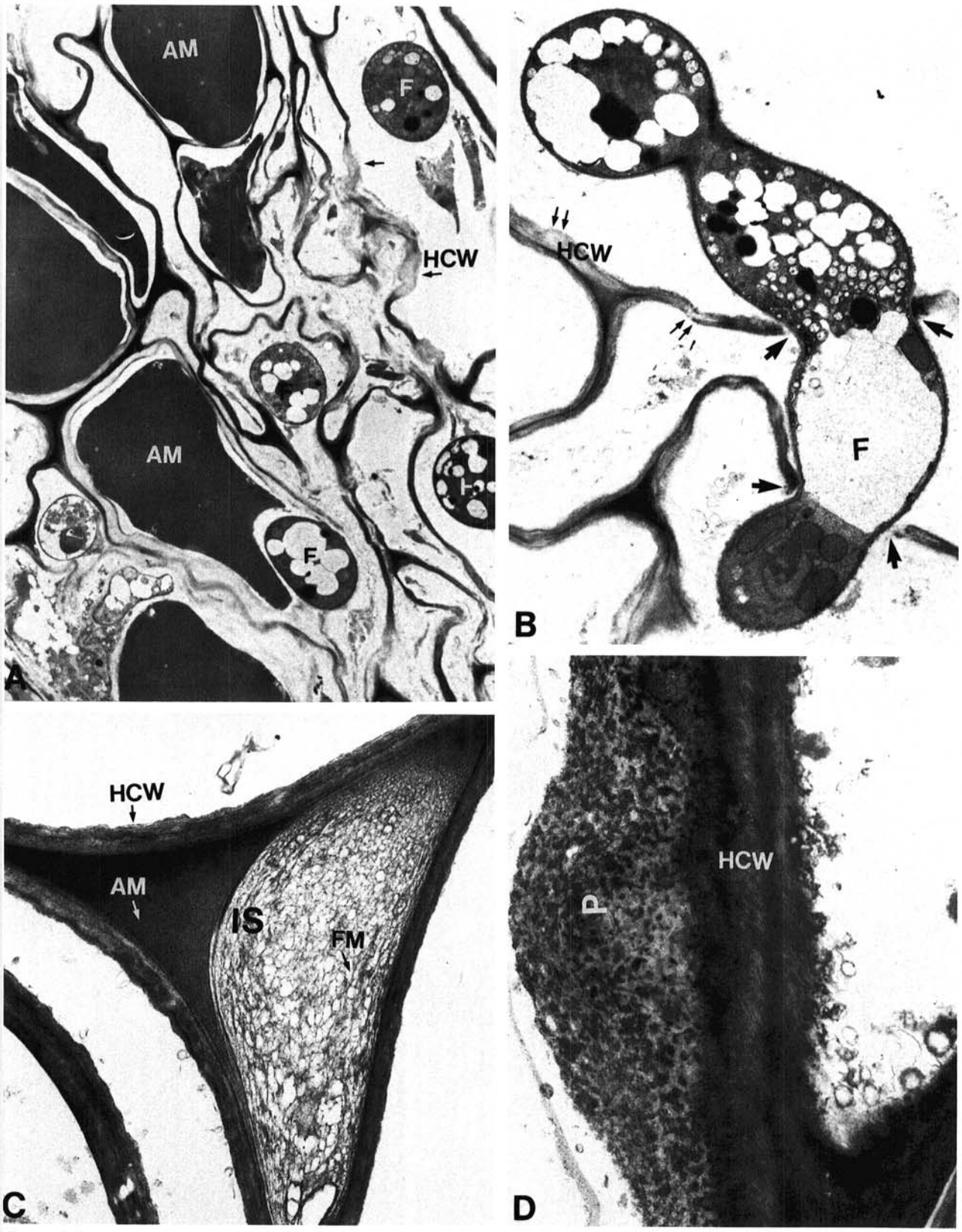

Fig. 5. Transmission electron micrographs of root tissue from cucumber plants grown in the presence of chitosan (0 or 400 μg/ml), 4 days after inoculation with *Pythium aphanidermatum*. **A and B,** Inoculated control plants. **A,** Massive fungal colonization of the cortex region. Pathogen ingress was followed by host cell-wall alterations (arrows). Some host cells were occluded with electron-opaque amorphous material (×3,200). **B,** Host cell-wall penetration by a fungal cell. There was no host cell-wall displacement in the direction of fungal growth (arrows) (×6,400). **C and D,** Chitosan-treated (400 μg/ml) plants. Various host reactions were stimulated by chitosan treatment: The most typical were **C,** plugging of intercellular space with electron-opaque and fibrillar material (×12,800) and **D,** oversized papilla formation along the host cell wall. The papilla appeared to be made of heterogeneous materials (×16,000). AM = Amorphous material; F = fungal cell; FM = fibrillar material; HCW = host cell wall; IS = intercellular space; P = papilla.

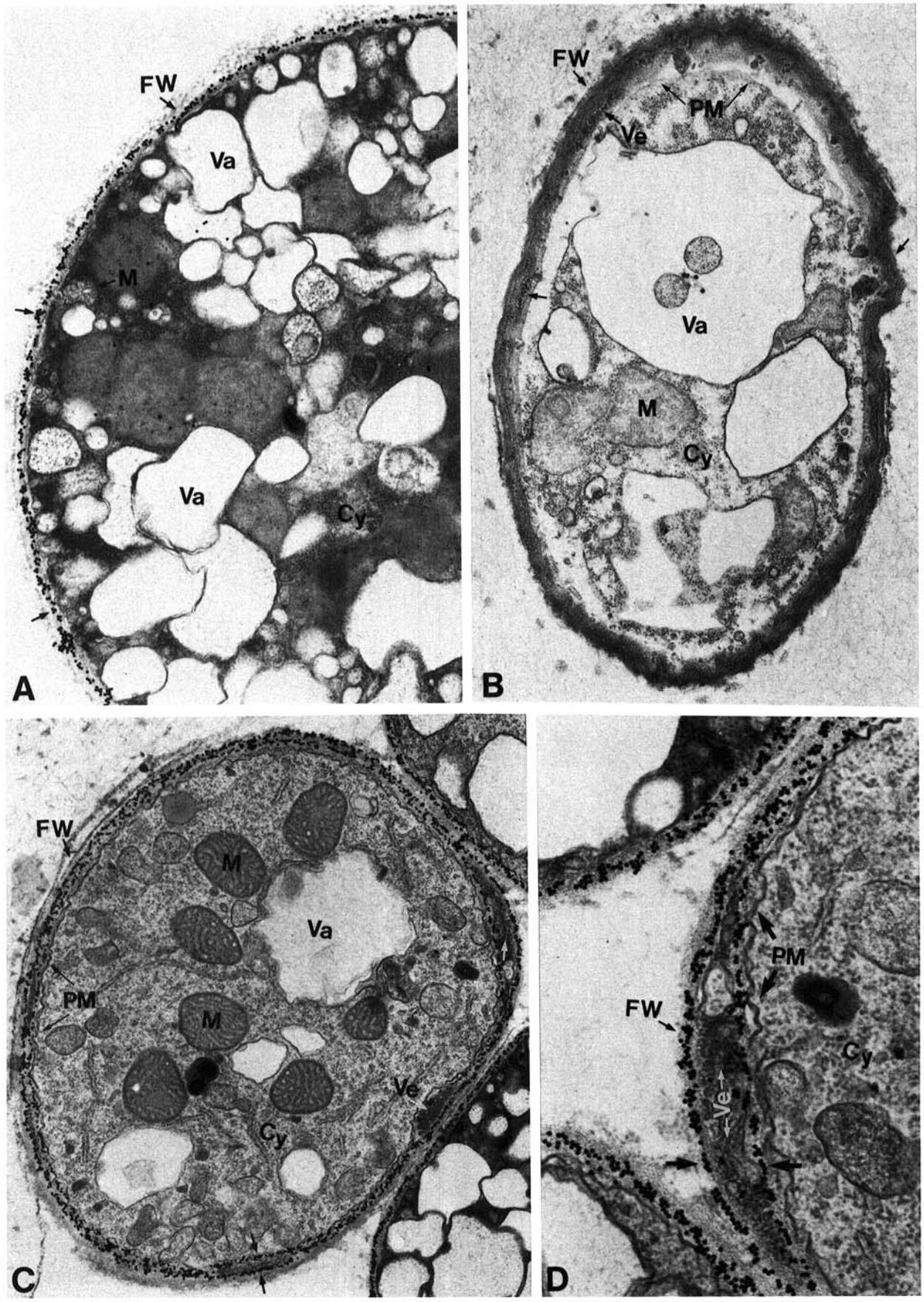

Fig. 6. Transmission electron micrographs of *Pythium aphanidermatum* grown on potato-dextrose agar (PDA) containing chitosan at 0 or 400 μg/ml. **A,** Control conditions (chitosan-free PDA). A cell of *P. aphanidermatum* delimited by a thin wall. Incubation with the gold-complexed exoglucanase resulted in the preferential deposition of gold particles over the innermost wall layer (arrows) (×24,000). **B–D,** Chitosan at 400 μg/ml. **B,** Vacuolated hyphal cell surrounded by a loosened wall (arrows). The plasma membrane was retracted from the cell wall and convoluted (arrows) (×12,800). **C and D,** Incubation with the gold-complexed exoglucanase resulted in the accumulation of gold particles over the loosened cell wall. Polymorphic inclusion vesicles were deposited in the paramural space (arrows) (**C,** ×16,000; **D,** ×40,000). Cy = Cytoplasm; FW = fungal wall; M = mitochondrion; PM = plasma membrane; Va = vacuole; Ve= vesicle.

dermal layer. The amendment of chitosan stimulated several host defense reactions in root tissues. In the cortex tissue, oversized papilla deposition frequently occurred along the host cell walls (Fig. 5D). The structure of these papillae appeared heterogeneous. In addition, the plugging of some intercellular spaces also was observed in both the cortical and endodermis tissues (Fig. 5C). In contrast, occlusion of parenchyma cells, similar to that observed in control plants, was seldom seen.

Antifungal activity of chitosan. Chitosan was effective in inhibiting mycelial growth of *P. aphanidermatum*. At a concentration of 400 μg/ml, chitosan completely inhibited the growth of *P. aphanidermatum*, whereas a chitosan concentration of 100 μg/ml caused 75% reduction of mycelial dry weight. In addition, chitosan appeared to affect the development of *P. aphanidermatum* in submerged culture. At concentrations below 400 μg/ml, *P. aphanidermatum* grew in the form of cell clusters, indicating that chitosan may have affected the process of hyphal extension.

To gain more information regarding the effect of chitosan on the growth of *P. aphanidermatum*, ultrathin sections from the fungus grown on PDA in the absence or presence of chitosan (0 or 400 μg/ml) were studied at the ultrastructural level (Fig. 6A–D). Under chitosan-free conditions, hyphal cells were delimited by a thin electron-opaque wall against which the plasma membrane was closely appressed (Fig. 6A). The presence of polysom-rich cytoplasm with numerous organelles, such as mitochondria and nuclei, suggested intense metabolic activity in these cells. Incubation of ultrathin sections with exoglucanase-gold complex resulted in a specific deposition of gold particles over the fungal cell wall (Fig. 6A). Labeling was associated mainly with the inner wall layer and did not occur over cytoplasm and other organelles (Fig. 6A, arrows).

Examination of ultrathin sections of *P. aphanidermatum* grown in the presence of chitosan revealed the occurrence of fungal cells at different degrees of disorganization, ranging from wall loosening to vacuolation and protoplasm disintegration. A localized deposition of polymorphic inclusion vesicles was frequently observed between the wall in the "paramural space" (Fig. 6C–D). In these cells, the plasma membrane was usually retracted from the cell wall and convoluted (Fig. 6D). In some cases, increased vacuolation associated with generalized plasmalemma retraction and protoplasm alteration was observed. Such cells generally were surrounded by loosened and distorted cell walls (Fig. 6B). After incubation with the exoglucanase-gold complex, gold particles also were associated specifically with the fungal cell wall (Fig. 6C–D). However, the inclusion vesicles lying in the paramural spaces were free of labeling, indicating that they did not originate from the cell wall. A close examination of numerous fungal cells revealed that the unlabeled vesicles were surrounded by gold particles that occurred on the cell wall side and in areas underlying the retracted plasma membrane. At these sites, the cell wall appeared to be split due to the new formation of vesicles (Fig. 6C–D, arrows).

DISCUSSION

In recent years, there has been an increasing interest in the use of chitosan as a speciality chemical in agriculture, and several potential biological functions have been identified (2,14,24). Attempts to exploit the antifungal and filmogenic properties of chitosan were recently carried out with postharvest produce. Chitosan when applied as a coating prolongs the storage life of fresh produce (16) and reduces the incidence of decay, an effect originating from its antifungal activity (14). In the present study, we present the first report that chitosan has the potential to suppress Pythium root rot of hydroponically grown cucumber plants.

Growing cucumber plants in the presence of chitosan not only controlled root rot caused by *P. aphanidermatum*, but also stimulated an array of defense mechanisms, such as physical barriers and defense enzymes, without affecting normal plant growth. Plants grown in the presence of chitosan did not display any symptoms of phytotoxicity even at higher chitosan concentrations, although changes in the morphology of secondary roots did occur. Although chitosan has been shown to induce membrane dysfunction in plant-cell suspension, as evaluated by leakage of proteinaceous and other UV-absorbing material, we did not observe a significant increase in leakage when plants were grown in the presence of chitosan. This is supported further by the healthy appearance of plants grown in the presence of chitosan. This is likely explained by the fact that our experiments were run with concentrations three-times lower than the one reported to induce severe leakage in plant cells (8).These observations are of key importance in relation to the nonphytotoxic nature of chitosan treatment at concentrations as low as 400 μg/ml. However, at high concentrations (>1,000 μg/ml) an adverse effect on plant growth can be anticipated. Even though chitosanase activity was detected in the root system, the analysis of nutrient solutions after SDS-PAGE revealed that chitosan was not hydrolyzed into oligomers.

In the present study, chitosan amendment of the nutrient solution stimulated chitinase, chitosanase, and β-1,3-glucanase activity in roots and leaves of cucumber plants. Elicitation of plant defense mechanisms by chitosan also has been demonstrated in other systems (14,24,26). In strawberry fruit, chitosan stimulated chitinase activity when applied directly to excised tissue (14). Increase in chitinase and β-1,3-glucanase activity also was reported in excised pea pods treated with chitosan (26). The observed increase of leaf glucanohydrolases triggered by the interaction of chitosan with the root system indicated a systemic effect of chitosan. Accumulation of glucanohydrolases with antifungal potential has been associated often with disease resistance in several plant-pathogen systems (6). However, it is unlikely that the glucanohydrolases present in roots could exert any effect on *P. aphanidermatum*, whose wall does not contain either chitin or chitosan. These hydrolytic enzymes are more likely to play a supporting role in resisting other pathogens. A synergistic increase in their activities may be important for their optimal function in plant resistance.

Chitosan amendment also induced various mechanical defensive reactions in cucumber tissues. Examination of root samples revealed that chitosan induced the formation of papillae impregnated with electron-opaque substances and, in some instances, the occlusion of intercellular spaces in noninfected epidermal cells. A similar deposition of material was observed in the cucumber-*P. ultimum* interaction (11) and was associated with the accumulation of phenolic-like compounds. Chérif et al (11) showed that penetration of the amorphous occluding material by *Pythium* hyphae often resulted in severe damage to the hyphal cells and even death. The importance of mechanical defensive structures in restricting fungal ingress has been shown in several other plant-pathogen systems (1). Although it is not possible to determine from the present data the extent of the role played by these physical barriers in preventing *P. aphanidermatum* invasion, they appear to have contributed to the restriction of infection by *P. aphanidermatum*. This is indirectly supported by the fact that only a few fungal cells were detected in the root tissue and that they were restricted mainly to the root surface. Some restricted fungal cells displayed alterations similar to those observed in vitro.

Results obtained from this study further indicate that disease suppression by chitosan can be attributed to some antifungal property of this compound. Indeed, chitosan was effective in inhibiting the growth of *P. aphanidermatum* in nutrient solutions. The inhibitory property of chitosan is well-known and has been demonstrated against several pathogenic fungi (2,13,15), including soilborne pathogens such as *P. paroecandrum* (25,31). Recently, chitosan was shown to cause severe morphological changes in *Rhizopus stolonifer* characterized by excessive branching and swelling of the cell wall (13) and to induce pronounced morphological alterations in *F. oxysporum* (4). Cellular and ultrastructural alterations also were observed in *P. aphanidermatum* treated with chitosan. Hyphal cells grown in the presence of chitosan showed alteration of the plasma membrane and modification of the cell walls, although the cellulose content did not seem to be affected. Such changes are likely to have a deleterious effect on virulence and growth of *P. aphanidermatum*. The role of the

vesicles formed in paramural spaces remains unknown. Whether these vesicles correspond to defense mechanisms stimulated in response to chitosan deserves further investigation.

In conclusion, this study demonstrates the potential of chitosan, a nontoxic compound, to suppress Pythium root rot of cucumber when used as an amendment in nutrient solutions of hydroponic systems. This potential value appears to be attributable to the combination of the antifungal and eliciting properties of chitosan. These unique properties may very well make chitosan an ideal antifungal agent against greenhouse diseases, considering its nontoxic effect on the environment.

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